Biosurfactant production and concomitant hydrocarbon degradation potentials of bacteria isolated from extreme and hydrocarbon contaminated environments

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Abstract

Amongst the total forty-seven bacterial isolates, eleven potent biosurfactant producing and concomitant hydrocarbon degraders were obtained after primary screening involving drop collapse method (DCM) and Oil-spreading method (OSM) followed by secondary screening comprising of Haemolytic assay (HA), Cetyl trimethyl ammonium bromide (CTAB) assay, Surface tension (ST), Emulsification index (E_{24}) and Emulsification activity (EA). 16S-rRNA sequencing and phylogenetic analysis revealed the presence of *Achromobacter, Bacillus*, *Citrobacter*, *Lysinibacillus, Ochrobactrum* and *Pseudomonas*. Two genera, *Achromobacter* (PS1) (observed for the first time) and *Bacillus* (SLDB1) were found to be glycolipid producers as evident by TLC, FT-IR and GC-MS chromatograms. The surface tension values were 30.43 mN/m and 31.10 mN/m with (E_{24}) of 69.90% and 65.23% respectively. Similarly the TLC, FT-IR and GC-MS results of the other two genera *Ochrobactrum* (GREW1) and *Bacillus* (SB2) confirmed them as lipopeptide biosurfactant producers with surface tension values of 31.14 mN/m and 28.16 mN/m and (E_{24}) of 59.51% and 61.35% respectively. Qualitative 2,6 - Dichlorophenol Indophenol (2,6 - DCPIP) and quantitative methods for hydrocarbon degradation revealed that *Achromobacter* sp. (PS1) showed a maximum degradation (46.32%) of 2% (w/v) crude oil with 70.77% and 77.17% reduction in peak area of aliphatic and aromatic fractions respectively with simultaneous lowering of surface tension from 59.27 mN/m (control) to 32.43 mN/m in 7 days. In case of *Achromobacter* sp. (PS1) and *Bacillus* sp. (SB2 and SLDB1), glucose supported biosurfactant production, whereas in *Ochrobactrum* sp. (GREW1) glucose along with 1% diesel enhanced biosurfactant production. This signifies the role of substrate in the nature of biosurfactants produced.

Keywords: Biosurfactant; Hydrocarbon degradation; Microbial Diversity; Glycolipid; Lipopeptide.

1. Introduction

Keeping in view of the increasing awareness towards environmental safeguards, stringent policies, volatile petroleum prices and simultaneous increase in consumer's demand, in recent years, the focus has been directed at the use of microbially produced surface active amphiphilic compounds known as the biosurfactants. They are promising substitutes for chemically – synthesized surfactants because of their unique properties like higher biodegradability, low toxicity, ecological acceptability, increased surface activities, higher foaming, low critical micelle concentrations (CMC), high selectivity and specificity at extreme temperatures, pH and salinity ranges. Biosurfactants constitute a heterogeneous group of biomolecules ranging from low molecular weight glycolipids, lipopeptides, flavolipids, phospholipids to high-molecular-weight polymers as lipoproteins, lipopolysaccharide-protein complexes and polysaccharide protein fatty acid complexes with wide structural and functional variability. These factors make them a multifunctional material of the 21st century with many commercial applications as cosmetics, personal care, textile processing, food, agricultural formulations, pharmaceutical industries, soil remediation, hydrocarbon degradation and oil recovery. For a bioremediation application, where solubility is an important criterion, biosurfactants prove to be promising vehicles for the removal of toxic polyaromatic hydrocarbons / dyes from contaminated soil / aquifers by lowering the surface tension and increasing the solubility of these compounds, thereby enhancing their bioavailability. In heavy metal-contaminated environments, biosurfactant enhances metal desorption from soils by forming complexes with free non-ionic forms of metals in solution. It also makes direct contact with absorbed metals at solid-solution interface under conditions of reduced interfacial tension with subsequent sequestration of metals into micelles [1].

Globally, in 2015 the biosurfactant market was estimated at 370.5 kilo tons, which is expected to reach 476.5 kilo tons equivalent to 2.21 billion USD by 2018 and to a further 2.69 billion USD by 2023 with a compound annual growth rate (CAGR) of 4.2% [2]. However, the successful commercialization of the biosurfactants is hindered by the high raw material and processing costs, lower product concentrations and severe stable foam formation under aerated and agitated conditions. These factors can be overcome by the selection of efficient strains, optimized medium composition, use of cost-free agro-industrial wastes, development of novel economical and efficient downstream processing methods. This study is designed to isolate and screen potent biosurfactant producing bacteria with hydrocarbon degrading potential.

2. Materials and methods

2.1. Chemicals

All chemicals, solvents and reagents used in the present study were of analytical grade. The crude oil was provided by IOCL Mathura refinery, Uttar Pradesh, India. Glycolipid (Rhamnolipid) standard (JBR 215, 15% solution in water) was obtained from Jeneil biosurfactant Company (Saukville, WI, USA), Lipopeptide standard (Surfactin) of 99.0% purity from Sigma.

2.2. Sampling sites

Hydrocarbon contaminated samples as oil spilled soil, raw oil effluent, activated sludge, refinery tank settled sludge, sludge with oil recovered, dry sludge, waste drain, refinery tank cleaning water, surge pond water and bioremediation site soil were collected from various sections of petroleum refineries as these are known to be excellent sources for isolating biosurfactant producing microbes. Also, the samples from extreme environments as desert soil and hot spring water were collected. The samples were collected in sterile Duran bottles and stored at 4^{0}C until use.

2.3. Enrichment and isolation

For isolation of pure microbial colonies, collected samples were subjected to three successive cycles of enrichment in erlenmeyer flasks containing 50 mL of minimal salt medium (MSM), pH 6.5 supplemented with 1% (v/v) of sterile diesel as carbon source and incubated at 30ºC, 120 rpm for 7 days. The composition of minimal salt medium (g/L) is as follows: $NaNO_3$ (7.5); KCl (1.1); NaCl (1.1); $FeSO_4.7H_2O$ (0.00028); K_2HPO_4 (4.4); KH_2PO_4 (3.4); $MgSO_4.7H_2O$ (0.5); yeast extract (0.5); glucose ($C_6H_{12}O_6$) (10.0) and trace element composition (g/L): $ZnSO_4.7H_2O$ (0.29); $CaCl_2.4H_2O$ (0.24); $CuSO_4.5H_2O$ (0.25); $MnSO_4.H_2O$ (0.17) [3].

The final enrichment culture broth was plated on Bushnell Haas (BH) agar plates supplemented with (1% v/v) diesel and incubated at 30ºC for 24 h. BH agar is a recommended medium for studying the microbial utilization of hydrocarbons. The composition of BH agar (g/L) is, $MgSO_4.7H_2O$ (0.2); $CaCl_2.4H_2O$ (0.02); KH_2PO_4 (1.0); K_2HPO_4 (1.0); NH_4NO_3 (1.0); $FeCl_3$ (0.05) and agar (20.0) [4]. Phenotypically different colonies were picked and pure colonies were maintained on nutrient agar (NA) plates with subsequent sub-culturing for further studies.

2.4. Screening of biosurfactant producing isolates

The phenotypically different colonies obtained were initially screened using drop collapse and oil spread method as these methods give more precise response based on the surfactant property. The stability of the drops and the displacement of oil are dependent on the surface tension and correlated to the biosurfactant concentration [5]. Subsequently, the colonies found positive in primary screening were further selected for secondary screening.

2.4.1. Primary screening:

2.4.1.1. **Drop collapse method (DCM):** Approximately 40 µL of culture filtrate was placed on the hydrophobic surface of parafilm strip to observe the shape of the drop. The presence of surfactant leads to a reduction in the interfacial tension between the hydrophobic film and

hydrophilic drop which cause the appearance of flat/collapsed drops on the film. Un-inoculated filtrate served as the negative control [5].

2.4.1.2. **Oil-spreading method (OSM)**: Approximately 30 mL of distilled water was poured to a petri dish of 90 mm diameter followed by the addition of 30 µL of diesel on the water surface to form a thin oil layer. Subsequently, 10 µL of culture supernatant obtained after seven days of incubation was gently placed on the center of the oil layer surface. The diameter of displaced oil was chosen as the criteria for the selection of potent biosurfactant producers [5].

2.4.2. Secondary screening:

2.4.2.1. **Haemolytic assay (HA):** Haemolytic assay was carried out by incubating the streaked 5% blood agar plates (Hi-Media) with overnight grown culture at 30ºC for three days [5]. Appearance of dark green zones under the colonies indicate alpha haemolysis or partial haemolysis. A yellow transparent zone around the colony indicate beta or complete hemolysis of the blood cells. No change in the blood agar plates indicates gamma or no hemolysis. Alpha and beta haemolysis were considered positive for biosurfactant production.

2.4.2.2. **Cetyl trimethyl ammonium bromide (CTAB) agar test**: Approximately 30 µL of each 72h cell-free culture supernatant was loaded into pre-cut wells in CTAB-methylene blue agar plates and incubated at 30°C for 72 h. The appearance of a dark blue halo zone around the well was considered positive for anionic biosurfactant production [6].

2.4.2.3. **Emulsification index (E_{24}):** In a 15 mL clear glass tube, 1 mL each of diesel and seventh day culture filtrate was added and vortexed vigorously for 2 min. The emulsified mixture was allowed to stand undisturbed at room temperature for 24 h to separate the aqueous and oil phases. The emulsification index (E_{24}) was calculated as the percentage of height (cm) of the emulsion layer divided by the total height (cm) [5].

92 ***2.4.2.4.*** **Emulsification activity (EA):** For emulsification activity, an emulsification mixture
93 comprising of 0.3 mL diesel, 1 mL of buffer and 0.5 mL of cell-free supernatant was incubated for
94 1h at room temperature and the absorbance of the aqueous layer was determined
95 spectrophotometrically at 400 nm. This constitutes the test sample (A_T) as against the control (A_C)
96 in which the cell-free supernatant was replaced by 0.5 mL distilled water. The dilution factor was
97 kept into consideration [7].

98 $$EU/mL = \text{Absorbance at } 400nm \text{ x dilution factor}/0.01 \qquad \textbf{Eq (A.1)}$$

99 **2*.4.2.5*. Surface Tension (ST)**: The surface tension of the culture filtrate obtained after seven days
100 of incubation was measured with a digital surface tensiometer (SEO, Instruments, Korea) working
101 on the principle of Du Nouy ring method [8]. An un-inoculated flask served as the control. The
102 lowering of surface tension is dependent on the surfactant property. All surface tension readings
103 were taken in triplicates.

104 **2.5. Identification of the biosurfactant producers**

105 **2.5.1. Biochemical characterization**

106 The bacterial isolates were characterized and identified using the results of following biochemical
107 tests - IMViC, catalase, coagulase, triple sugar iron (TSI), arginine dihydrolase, casease, gelatin
108 hydrolysis as recommended by Bergey's manual of determinative bacteriology and as described
109 in "Laboratory exercises in microbiology" [9,10].

110 **2.5.2. Molecular identification**

111 ***2.5.2.1.*** **DNA extraction and PCR amplification of 16S-rRNA gene fragment**

112 The bacterial isolates were identified by 16S-rRNA gene sequencing. Genomic DNA of the
113 bacterial isolates was extracted from overnight grown culture, the extracted genomic DNA was
114 used as the template for PCR amplification of the 16S-rRNA sequence of the isolates using two

115 universal primers. These were BS1F 5′-GAGTTTGATCCTGGCTCA-3′ and BS1R
116 5′ACGGCTACCTTGTTACGACTT-3′, which are complementary to the conserved regions at the
117 5′- and 3′- ends of the 16S-rRNA gene corresponding to positions 9-27bp and 1477-1498bp of the
118 *Escherichia coli* 16S-rRNA gene [11]. The thermal cycle amplification program was performed
119 on a Bio-Rad PCR system 2400 (Bio-Rad laboratories, USA) with temperature program as: 94ºC
120 for 5 min, 94ºC for 1 min, 54ºC for 1 min, 72ºC for 1 min, 30 cycles; then final extension at 72ºC
121 for 10 min and finally storage at 4ºC. The purity and size of each PCR product was examined by
122 resolving the amplified product by gel electrophoresis on 2.0% agarose gel in 1× TAE (Tris acetate
123 EDTA) buffer for 1–2 h at 80V. The gel profiles were visualized in UV gel documentation system
124 (Bio-Rad laboratories, USA).
125 The sequencing of the amplified 16S-rRNA gene was outsourced from Eurofins genomics India
126 Pvt. Ltd. Bangalore. The amplified 16S-rRNA gene was sequenced through Sanger sequencing
127 method in Applied Biosystems 3730xL Genetic Analyzers, USA. The sequenced nucleotides were
128 searched for 16S-rRNA gene sequence homology using BLAST algorithm with NCBI database
129 [12].
130 Multiple sequence alignment was performed using CLUSTAL W and the phylogenetic tree was
131 constructed using the Neighbor-Joining method. The evolutionary distances were computed using
132 the maximum composite likelihood method and were expressed in the units of the number of base
133 substitutions per site. Phylogenetic analyses were conducted using MEGA4 [13].
134 The 16S-rRNA sequences of isolates from this study have been submitted to the NCBI Genbank
135 database and the accession numbers have been provided for the same.
136 **2.6. Evaluation of hydrocarbon degrading potential**

One of the numerous adaptations of microorganisms induced by presence of hydrocarbons is biosurfactant production, which helps in metabolizing and degrading hydrocarbons thereby representing a physiological response [14]. Hence, the eleven selected isolates obtained after secondary screening were also evaluated for hydrocarbon degradation potential both qualitatively and quantitatively.

2.6.1. Qualitative degradation using Di-chlorophenol indophenol (DCPIP) method

Hydrocarbons ranging from simple to complex with varying chain lengths were selected to evaluate the hydrocarbon degrading potential of the isolates using DCPIP as redox indicator in W medium with hydrocarbon source at 1% (v/v) [15]. These were hexane, decane, benzene, diesel (C10 - C19 hydrocarbons) and crude oil (mixture of straight or branched chain aliphatics ranging from C4 - C40 and aromatics from benzene to multi-ring polycyclic compounds). The acceptance of electrons by an electron acceptor (DCPIP) results in the change of redox state from oxidized (blue) to reduced state (colourless) thereby indicating the use of the hydrocarbon as carbon source.

2.6.2. Quantitative degradation using gravimetric method and GC-FID

Gravimetric method

Quantitatively the degradation of the hydrocarbon, in crude oil was evaluated by inoculating overnight grown culture (OD_{600} equivalent to 1.0) of these respective eleven isolates separately in 50 mL of MSM supplemented with 2% (w/v) crude oil as sole carbon source at 30°C, 120 rpm for seven days. Un-inoculated medium was treated as control. After seven days of incubation, the culture broth was filtered through a porous layer of non-absorbent cotton to separate the crude oil from the growth medium. The filtered broth was then centrifuged at 5000 rpm, 4°C and the supernatant was analyzed for surface tension measurements. Each treatment was performed in triplicates [16].

160 The residual amount of the total petroleum hydrocarbon (TPH) in the flask and the cotton was
161 recovered by adding 20 mL of petroleum ether thrice. The petroleum ether fraction containing the
162 residual crude oil of each treatment was pooled and dehydrated using anhydrous sodium sulfate
163 for 4 h to remove moisture. This was then vacuum dried using a rotary evaporator at 40ºC. The
164 mass of the residual oil was measured by gravimetric method using a high accuracy electronic
165 balance (Sartorius, Germany). The degrading efficiency of the isolates was calculated as follows
166 [17],

167 $$\text{TPH degrading efficiency} = [1 - (Y + e) / X] * 100 \qquad \textbf{Eq (A.2)}$$

168 Where '*X*' is initial crude oil concentration (g)
169 '*Y*' is residual oil (g)
170 '*e*' is oil loss (g) due to evaporation, [Oil loss = weight of crude oil in control (uninoculated)
171 flask at zero day of incubation - weight of crude oil in control (uninoculated) flask after seven
172 days of incubation]

173 **Gas Chromatography -FID Analysis**

174 Approximately 0.1g of degraded TPH was dissolved in *n*-pentane to remove all asphaltenes;
175 subsequently, the soluble fraction was air dried and re-dissolved in 1 mL of hexane and loaded on
176 silica gel (60-120 mesh) column. Aliphatic and aromatic fractions of the crude oil were separated
177 by eluting the loaded sample using hexane (150 mL) and toluene (150 mL) respectively [18]. The
178 hexane and toluene extracted fractions were further vacuum dried and analyzed by gas
179 chromatography (GC) (Shimadzu GC-2010 7890A) with flame ionization detector (FID) using
180 capillary Rtx-5MS column (25 m × 0.25 mm × 0.25 um) to examine the degradation profile of
181 crude oil by these eleven isolates. The oven temperature program was: initial temperature: 110ºC

held for 2 min, then heated to 250ºC at 10ºC/min and held for 5 min, then heated to 280ºC at 15ºC/min and held for 17 min. Nitrogen was used as carrier gas (28.4 mL/min).

2.7. Biosurfactant production profile for four best biosurfactant producers

Based on the biosurfactant production and hydrocarbon degradation profile, four organisms were selected to evaluate the biosurfactant production pattern in terms of supplementation of carbon source in MSM. The experiment involved three sets; A: 1% (w/v) dextrose supplemented with 1% (v/v) diesel; B: 1% (w/v) dextrose only and C: 1% (v/v) diesel only.

2.8. Extraction of biosurfactant

The culture filtrate obtained was acidified with 6N HCl to pH 2.0 and kept overnight for precipitation. The precipitate was separated after centrifugation and extracted twice with a mixture of chloroform: methanol (2:1 v/v). The extracts were pooled and were concentrated under vacuum using a rotary evaporator [16].

2.9. Characterization of the partially purified biosurfactant

2.9.1. Thin layer chromatography

The partially purified extract was analyzed by thin layer chromatography (TLC) on silica gel 60 F_{254} plates (Merck Co., Inc., Damstadt, Germany) with a solvent system consisting of chloroform/methanol/water (65:15:2, v/v/v) and visualized with different color developing reagents. Ninhydrin reagent (0.2% ninhydrin in ethanol) was used to detect lipopeptide biosurfactant as red-pink spots, iodine vapour for detecting lipids, orcinol-sulphuric acid reagent (1% in concentrated sulphuric acid) for the presence of glycolipids which shows the appearance of brown spots on heating to 110ºC [16].

2.9.2. Fourier transform infrared (FT-IR) spectroscopy

204 Fourier transform infrared spectroscopy (FT-IR) analysis for molecular composition of
205 biosurfactant was carried out using a Varian-7000 Fourier transform-infrared spectrophotometer
206 by first preparing its pellet in spectral grade KBr applying 5–6 tons /cm^2 of pressure for 10 min
207 using the hydraulic press followed by its scanning in the transmittance mode in the range of 400–
208 4000 cm^{-1} with a resolution of 4 cm^{-1} [12].

209 **2.9.3. Gas chromatography–mass spectrometry (GC–MS) of fatty acids**

210 The partially purified biosurfactant (10 mg) was dissolved and derivatized in 1 mL of 1M HCl–
211 methanol using a closed screw-cap tube for 4 h at 100ºC. The product containing the fatty acid
212 methyl esters (FAMEs) was partitioned by adding double distilled H_2O (1 mL) and the organic
213 phase was extracted over anhydrous sodium sulfate for moisture removal. The samples were
214 analyzed by GC-MS: 1 µl was injected into a Shimadzu GC–MS (QP2010 ultra) equipped with a
215 RTX-5MS fused silica capillary column (0.25 mm film thickness, 0.25 mm internal diameter, 30
216 m in length). Helium at 1.5 mL/min flow rate was used as the carrier gas and the operating
217 temperature of the column and injector were 140ºC and 260ºC respectively, the electron impact
218 ion source was maintained at 230ºC. Electron impact mass spectra were recorded at 70 keV. The
219 mass spectrum of each fatty acid methyl ester was matched to the "National Institute of Standards
220 and Technology" (NIST) mass spectral library database to determine probable fatty acids
221 composition of the biosurfactant.

222 **3. Results and Discussion**

223 **3.1. Screening and identification of biosurfactant producing isolates**

224 Amongst forty-seven total isolates, thirty-seven isolates showed positive results for DCM and
225 OSM respectively in primary screening (Table 1). Of these thirty-seven primary screened colonies,
226 eleven colonies were finally selected based on secondary screening results which involved

lowering of surface tension in the range between 28 to 42 mN/m with the maximum reduction observed in *Bacillus* sp. (SB2), *Achromobacter* sp. (PS1), *Bacillus* sp. (SLDB1) and *Ochrobactrum* sp. (GREW1) with values of 28.16 ± 0.19 mN/m, 30.43 ± 0.44 mN/m, 31.10 ± 0.71 mN/m and 31.14 ± 0.68 mN/m respectively as against the un-inoculated (control) medium with a surface tension of 69.84 ± 2.2 mN/m and deionized water of 70.31 ± 1.84 mN/m (Table 2). Cooper [19] reported that an isolate can be a promising biosurfactant producer if it reduces the surface tension of a liquid medium to 40 mN/m or less. The highest emulsification index (E_{24}) of 69.90 ± 0.97%, 65.23 ± 1.48%, 64.22 ± 1.44% and 61.35 ± 1.15% was observed in isolates *Achromobacter* sp. (PS1), *Bacillus* sp. (SLDB1), *Pseudomonas* sp. (MRBSIT1), and *Bacillus* sp. (SB2) respectively with stable emulsions even after one week. The similar rating was observed for emulsification activity with highest in *Achromobacter* sp. (PS1) (97.05 ± 1.62 EU/mL). These colonies also showed mucoid colony morphology on BH agar plates supplemented with diesel indicating the production of exopolysaccharides as reported by Subudhi et al. [20].

Among the eleven isolates, five isolates were found to be Gram negative and six were Gram positive. The sequence and the phylogenetic analysis are shown in Fig. 1. The 16S-rRNA gene revealed the presence of six different genera *Achromobacter, Bacillus*, *Citrobacter, Lysinibacillus*, *Ochrobactrum* and *Pseudomonas*. These genera belonged to either Proteobacteria or Firmicutes phylum (Table 3). The 16S-rRNA gene sequence analysis showed 100% sequence similarity for *Pseudomonas* sp. (MRBSIT1) and *Ochrobactrum* sp. (GREW1), whereas 99% for all the other isolates. Occurrence of *Pseudomonas* as the dominant genera for biosurfactant production in hydrocarbon contaminated environments has also been reported by Chirwa and Bezza [21]. Saisa-Ard et al. [22] reported species belonging to *Acinetobacter*, *Bacillus*, *Corynebacterium*, *Pseudomonas*, *Rhodococcus* and *Serratia* as the most widely reported genera for biosurfactant-

producing bacteria from hydrocarbon contaminated terrestrial or marine sites, however only very few reports have been highlighted related to biosurfactant production from genera *Achromobacter* [23], *Citrobacter* [24], *Lysinibacillus* [25] and *Ochrobactrum* [26].

3.2. Evaluation of the hydrocarbon degrading potential of biosurfactant producers

Evaluation of qualitative hydrocarbon degradation using DCPIP by the eleven isolates of genera *Achromobacter*, *Bacillus*, *Citrobacter*, *Lysinibacillus*, *Ochrobactrum,* and *Pseudomonas* showed positive results for 1% (v/v) crude oil and diesel (Table 4). Of all the genera, only *Pseudomonas* (MRBSIT1) and two isolates of *Bacillus* (TC2 and SB2) were found to degrade hexane. For benzene, *Pseudomonas* (MRBSIT1) and *Citrobacter* (BRRO1) showed positive results. This limitation in degradation behavior with respect to hexane and benzene hydrocarbons may be explained on the basis of log K_{ow} (octanol/water partition coefficient), which proves to be a useful parameter to predict the suitability of the solvent for bioconversion [27]. It is well established that the solubility of hydrocarbons in aqueous system decrease while their respective log K_{ow} values increase with the corresponding increase in the molecular weight of hydrocarbons. This allows the diffusion of low molecular weight hydrocarbons hexane and benzene having low log K_{ow} values of 2.3 and 3.5 respectively from the surrounding aqueous system to the cells thereby destabilizing the cell membrane integrity and thus resulting in the limitation of hydrocarbon degradation. On the other hand, the higher molecular weight hydrocarbons (decane and diesel) with low solubility in aqueous system and corresponding higher K_{ow} values of 5.6 and 7.7 respectively form a biphasic system with the W medium used in DCPIP method. In the presence of these less soluble hydrocarbons, the logarithmic phase cells with cell density of 1.0 at OD_{600} nm, shows an adaptation by producing biosurfactant. These amphiphilic biosurfactant molecules partition themselves at the hydrocarbon water interphase and acts as a mediator by increasing the mass transfer rates of

hydrophobic contaminants (hydrocarbon based substances) into the aqueous phase through specific interaction resulting in mobilization and solubilization. Mobilization involves the reduction of surface and interfacial tension while solubilization results in the dramatic increase in the apparent solubility of hydrocarbon due to its aggregation within the surfactant micelles [28]. Similarly, Kubota et al. [15] reported degradation of decane using DCPIP method by one *Bacillus* (ODMI57) and two *Pseudomonas* sp. (F721 & F722). While none of the isolates amongst the thirty-six bacterial colonies belonging to different genera *Acinetobacter*, *Rhodococcus*, *Gordonia*, *Pseudomonas*, *Ralstonia*, *Bacillus* and *Alcaligens* were able to degrade benzene.

The gravimetric results of the residual total petroleum hydrocarbon (TPH) showed 46.32% degradation of 2% (w/v) crude oil by *Achromobacter* sp. (PS1) with surface tension value of 32.43 ± 0.83 mN/m, followed by 38.93% degradation by *Ochrobactrum* sp. (GREW1) with surface tension value of 33.14 ± 0.54 mN/m respectively as against the un-inoculated control (59.27 ± 1.16 mN/m). The GC-FID results of the percentage degradation of the aliphatic fraction by *Achromobacter* sp. (PS1) and *Ochrobactrum* sp. (GREW1) was observed as 70.77% and 79.24% respectively and that of aromatic fraction as 77.17% and 72.13% respectively (Table 4). Mnif et al. [29] also reported degradation of aliphatic fractions of 1% crude oil from micro-organisms of different genera with maximum degradation of 96.20% and 93.30% by *P. aeruginosa* and *H. lutea* in twenty days. Microbial degradation of crude oil has been shown to be fast in aerobic conditions as compared to anaerobic and occurs by the action of oxygenases on the aliphatic and aromatic fractions. Aerobic degradation of aliphatic hydrocarbons results in the formation of fatty acids. The carboxylic acid groups in the fatty acids are then further metabolized *via* the β-oxidation pathway to form acetyl CoA or propionyl CoA depending on the number of carbon atoms (even or odd) in the *n*-alkane. These compounds are then subsequently metabolized *via* the tricarboxylic

296 acid (TCA) cycle to CO_2 and H_2O, together with the production of electrons in the electron
297 transport chain. This chain is repeated, further degrading the aliphatic hydrocarbons. In aromatic
298 hydrocarbons, under aerobic conditions arenes in PAH rings are oxidized leading to formation of
299 intermediates such as protocatechuate and catechols. The catechols are further cleaved to
300 metabolites such as acetate, succinate, pyruvate or acetaldehyde, which subsequently enter the
301 TCA cycle and are thus available as energy and carbon sources to the cell [30]. On the other hand,
302 *Bacillus* sp. (SB2) though showed maximum reduction in surface tension of 28.16 mN/m in 2%
303 crude oil, yet the degradation percentage of TPH was found to be 20.91%. This may be explained
304 on the basis that the biosurfactant produced by *Bacillus* sp. (SB2) was not that efficient to emulsify
305 and utilize the extremely complex high molecular weight recalcitrant carbon structures of
306 asphaltenes present in crude oil due to its relatively lower emulsification index (E_{24}) of 61.35%.
307 Also, it has been reported that asphaltenes are recalcitrant to degradation [31] while NSO (nitrogen,
308 sulphur, and oxygen) compounds show an inhibitory effect on the degradation of many creosote
309 compounds comprising of six major classes – aromatic hydrocarbons (polyaromatic hydrocarbons
310 and alkylated polyaromatic hydrocarbons), tar acids/phenolics, tar bases/nitrogen containing
311 heterocycles, aromatic amines, sulphur containing heterocycles and oxygen containing
312 heterocycles [32]. *Pseudomonas* sp. (MRBSIT1) showed the preferential percentage degradation
313 of aliphatic fraction of crude oil to 93.86% though with a low TPH degradation of 25.19%. These
314 differences in the ability of the bacteria to degrade hydrocarbons may also be linked to several
315 other inherent factors such as cell surface hydrophobicity, catabolic enzyme activity, gene
316 arrangement besides the nature of the biosurfactant production [33]. Thus, this study gives a clear
317 association between the synthesis of emulsifying agent and concomitant hydrocarbon degradation

with solubilization of hydrocarbons rendering them more accessible for their breakdown and uptake.

3.3. Biosurfactant production profile of best biosurfactant producing and concomitant hydrocarbon degrading isolates

The biosurfactant production profile of the isolates *Achromobacter* sp. (PS1) and *Bacillus* sp. (SLDB1) showed low surface tension values of 32.12 ± 0.78 mN/m and 30.54 ± 0.70 mN/m respectively in set B comprising of only glucose against the set A comprising of glucose and diesel (Fig. 2). *Bacillus* sp. (SB2) showed almost same surface tension values (28.41 ± 0.41 and 28.52 ± 0.26 mN/m) in sets A and B. While *Ochrobactrum* sp. (GREW1) showed a different pattern of biosurfactant production with low surface tension values of (31.06 ± 0.28 and 31.38 ± 1.36 mN/m) in set A and C comprising of glucose with 1% (v/v) diesel and only 1% (v/v) diesel respectively as against the set B (only glucose) with surface tension of 56.75 ± 0.38 mN/m. This difference in production pattern may be attributed owing to the involvement of complex synthetic machinery governed by several intrinsic and extrinsic interacting parameters and also on the substrate composition.

3.4. Characterization of the biosurfactant

3.4.1. Thin layer chromatography (TLC)

The TLC result of the extracted biosurfactant from *Achromobacter* sp. (PS1) and *Bacillus* sp. (SLDB1) suggested a glycolipid nature of the biosurfactant with two prominent spots at R_f of 0.34 and 0.72 relating to di-rhamnolipid and mono-rhamnolipid moieties similar to Jeneil JBR 215 rhamnolipid standard when developed with orcinol reagent (Fig. 3A). Similar results were reported by Bhat et al. [34] for Jeneil standard rhamnolipid as well as rhamnolipid produced from *Pseudomonas aeruginosa* with the following R_f 0.35 and 0.73 values. Till date glycolipid

production has not been reported from *Achromobacter* sp. The genus *Bacillus*, has been most commonly reported for the production of lipopeptide type of biosurfactants [12]. Our results are however contrary to earlier reports as production of glycolipid type of biosurfactant was observed from *Bacillus sp.* (SLDB1) using 1% glucose but are in accordance with the reports of Chandankere et al. [35] wherein the production of glycolipid biosurfactant from *Bacillus methylotrophicus* USTBa has been reported using 2% crude oil. The TLC results for extracted biosurfactant from *Ochrobactrum* sp. (GREW1) and *Bacillus* sp. (SB2) showed positive spots when developed with ninhydrin reagent indicating the presence of peptide moieties in the molecule confirming the lipopeptide nature of the biosurfactant. The peptide moiety was observed as a single spot (R_f 0.55) in *Ochrobactrum* sp. (GREW1) and two spots (R_f 0.72 and 0.55) in case of *Bacillus* sp. (SB2) as shown in Fig. 3B. Similar TLC pattern was reported by Qiao and Shao [36] for lipopeptide type biosurfactant.

3.4.2. Fourier transform infrared (FT-IR) spectroscopy

Characteristic absorption bands corresponding to specific functional groups present in each of the four biosurfactants were determined and confirmed to characterize the type of biosurfactant. In *Achromobacter* sp. (PS1) and *Bacillus* sp. (SLDB1), a significant broad band at 3423 cm^{-1} and 3416 cm^{-1} respectively corresponds to O–H stretching vibrations of free hydroxyl groups and the stretching bands around 1080 cm^{-1} indicates the presence of polysaccharide or polysaccharide-like substances revealing the glycolipid nature of biosurfactants. Similar absorption bands were reported by Singh and Tiwary [37].

On the other hand, respective characteristic absorbance bands at 3275 cm^{-1} and 3277 cm^{-1}, resulting from the N-H stretching mode and the absorption bands corresponding to C–O stretching and N–H bending of amide groups around 1536 cm^{-1} and 1658 cm^{-1} in *Ochrobactrum* sp. (GREW1) and

364 *Bacillus* sp. (SB2) signifies the presence of peptide bonds and lipopeptide nature of the
365 biosurfactant. Ramani et al. [38] also reported that the characteristic stretching frequency of amides
366 which lie in the region 3250-3300 cm^{-1} and 1500-1650 cm^{-1} is specific for lipopeptide type of
367 biosurfactant and generally not observed in glycolipid biosurfactants.

368 **3.4.3. GCMS of Fatty acids**

369 The fatty acid compositions of the biosurfactant produced from *Achromobacter* sp. (PS1) and
370 *Bacillus* sp. (SLDB1), showed the presence of 3-hydroxydecanoic acid ($C_{10:0}$) as the most
371 abundant fatty acid with 88.27 ± 0.07 and 90.11 ± 0.07 relative percentage respectively. Other
372 fatty acids were also found however in minor amounts and were identified as 3-hydroxydodecanoic
373 ($C_{12:0}$), 3-hydroxytetradecanoic ($C_{14:0}$), 3-hydroxyhexadecanoic ($C_{16:0}$), 3-hydroxyoctadecanoic
374 ($C_{18:0}$), and 3-hydroxyheneicosanoic ($C_{21:0}$). A similar pattern was observed in the fatty acid
375 composition of rhamnolipid standard (Jeneil biosurfactant) with 3-hydroxy decanoic fatty acid as
376 the major (91.45 ± 0.04%) component (Table 5). This result of our finding is in accordance with
377 the reported literature for glycolipids where decanoic acid is the most commonly reported fatty
378 acid [39].

379 In case of *Ochrobactrum* sp. (GREW1) and *Bacillus* sp. (SB2), the fatty acid content of the
380 lipopeptides was diverse with several longer chain fatty acids. Major fatty acid composition peaks
381 of 3-hydroxyoctadecanoic acid (33.03 ± 0.09%) and 3-hydroxyheneicosanoic acid (32.82 ± 0.03%)
382 in *Ochrobactrum* sp. (GREW1) and 3-hydroxyhexadecanoic acid (56.89 ± 0.01%) and
383 hydroxyheneicosanoic (39.02 ± 0.03%) in *Bacillus* sp. (SB2) were observed. This diversity
384 observed in lipopeptide production may be a consequence of differences the fatty acid components
385 which not only depends on the producing bacteria but also on the culture conditions and substrates
386 used. A mixture of β-hydroxy fatty acids of dodecanoic, tetradecanoic, pentadecanoic,

hexadecanoic, octadecanoic, (9)-octadecenoic and (9,12)-octadecadienoic in the lipoprotein biosurfactant have also been reported by Qiao and Shao [36], Ramani et al. [38] and Leon et al. [40].

4. Conclusions

The potent biosurfactant producers and concomitant hydrocarbon degraders obtained from the present investigation provides an insight for the productive competence of different types of biosurfactants with *Achromobacter* sp. (PS1) and *Bacillus* sp. (SLDB1) producing glycolipids and *Ochrobactrum* sp. (GREW1) and *Bacillus* sp. (SB2) producing lipopeptides as confirmed by TLC, FT-IR and GC-MS results. All the biosurfactants exhibited high emulsification activity with low surface tension values and efficiently degraded crude oil revealing their promising applicability in bioremediation processes. Moreover, the utilization of cheap glucose as the carbon source in the case of *Achromobacter* sp. (PS1) and *Bacillus* sp. (SLDB1 and SB2) further directs towards the use of agro-waste residues for sustainable cost effective biosurfactant production with high yields.

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Legends of the Figures:

Fig. 1. Consensus neighbor-joining phylogenetic tree based on 16S-rRNA gene sequences showing the phylogenetic positions of eleven biosurfactant producing isolates selected in this study and published biosurfactant isolates from literatures. Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1000 replicates. The scale bar indicates substitutions per nucleotide. The GenBank accession numbers for the 16S-rRNA gene sequences are given in parentheses after the strain name.

Fig. 2. Biosurfactant production profile (30ºC, 120 rpm, 7 days):
(**A**) *Achromobacter* sp. (PS1), (**B**) *Bacillus* sp. (SLDB1),
(**C**) *Ochrobactrum* sp. (GREW1) and (**D**) *Bacillus* sp. (SB2).
The standard deviations presented are based on triplicate (SD, n = 3)

Fig. 3. TLC chromatograms:
(A) Glycolipid biosurfactant (**a**, **b** & **c**): **a**- Jeneil JBR -215 (standard);
b- *Achromobacter* sp. (PS1); **c**- *Bacillus* sp. (SLDB1)
(B) Lipopeptide biosurfactant (**d** & **e**): **d**- *Ochrobactrum* sp. (GREW1);
e- *Bacillus* sp. (SB2)

Fig. 1.

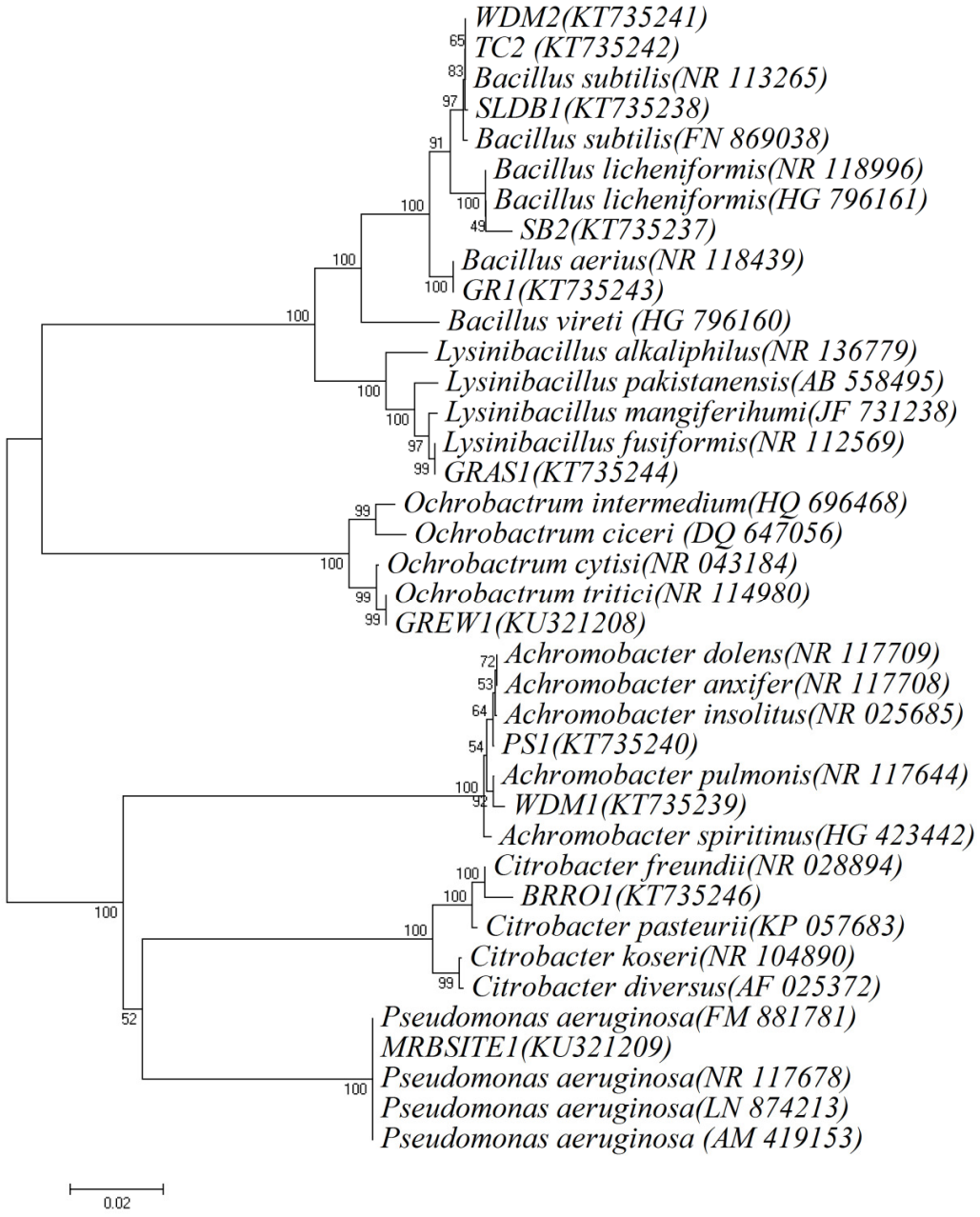
WDM2(KT735241)
TC2 (KT735242)
Bacillus subtilis(NR 113265)
SLDB1(KT735238)
Bacillus subtilis(FN 869038)
Bacillus licheniformis(NR 118996)
Bacillus licheniformis(HG 796161)
SB2(KT735237)
Bacillus aerius(NR 118439)
GR1(KT735243)
Bacillus vireti (HG 796160)
Lysinibacillus alkaliphilus(NR 136779)
Lysinibacillus pakistanensis(AB 558495)
Lysinibacillus mangiferihumi(JF 731238)
Lysinibacillus fusiformis(NR 112569)
GRAS1(KT735244)
Ochrobactrum intermedium(HQ 696468)
Ochrobactrum ciceri (DQ 647056)
Ochrobactrum cytisi(NR 043184)
Ochrobactrum tritici(NR 114980)
GREW1(KU321208)
Achromobacter dolens(NR 117709)
Achromobacter anxifer(NR 117708)
Achromobacter insolitus(NR 025685)
PS1(KT735240)
Achromobacter pulmonis(NR 117644)
WDM1(KT735239)
Achromobacter spiritinus(HG 423442)
Citrobacter freundii(NR 028894)
BRRO1(KT735246)
Citrobacter pasteurii(KP 057683)
Citrobacter koseri(NR 104890)
Citrobacter diversus(AF 025372)
Pseudomonas aeruginosa(FM 881781)
MRBSITE1(KU321209)
Pseudomonas aeruginosa(NR 117678)
Pseudomonas aeruginosa(LN 874213)
Pseudomonas aeruginosa (AM 419153)
0.02

Fig. 2.

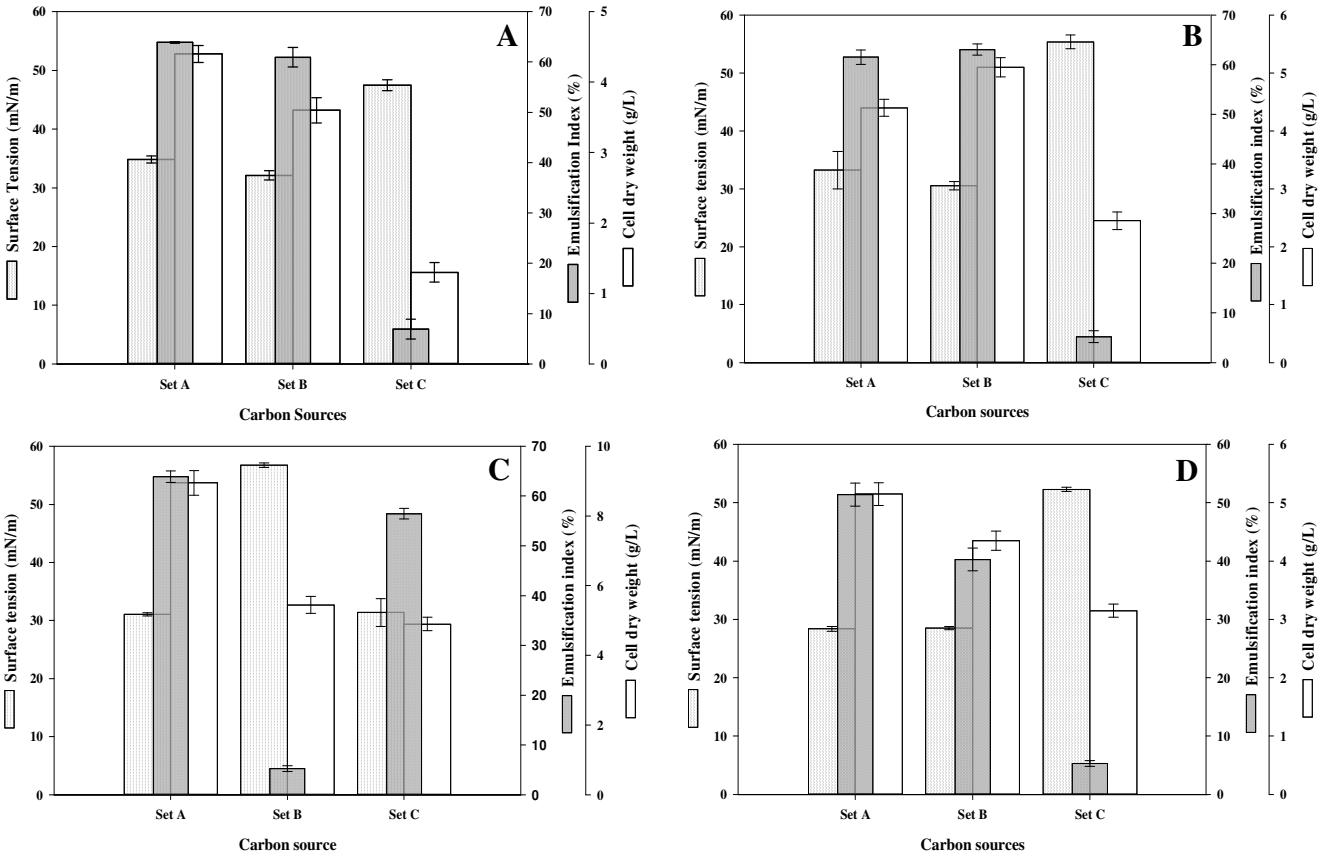
A
Surface Tension (mN/m)
Emulsification Index (%)
Cell dry weight (g/L)
Set A
Set B
Set C
Carbon Sources
B
Surface tension (mN/m)
Emulsification index (%)
Cell dry weight (g/L)
Carbon sources
C
Carbon source
D

Fig. 3

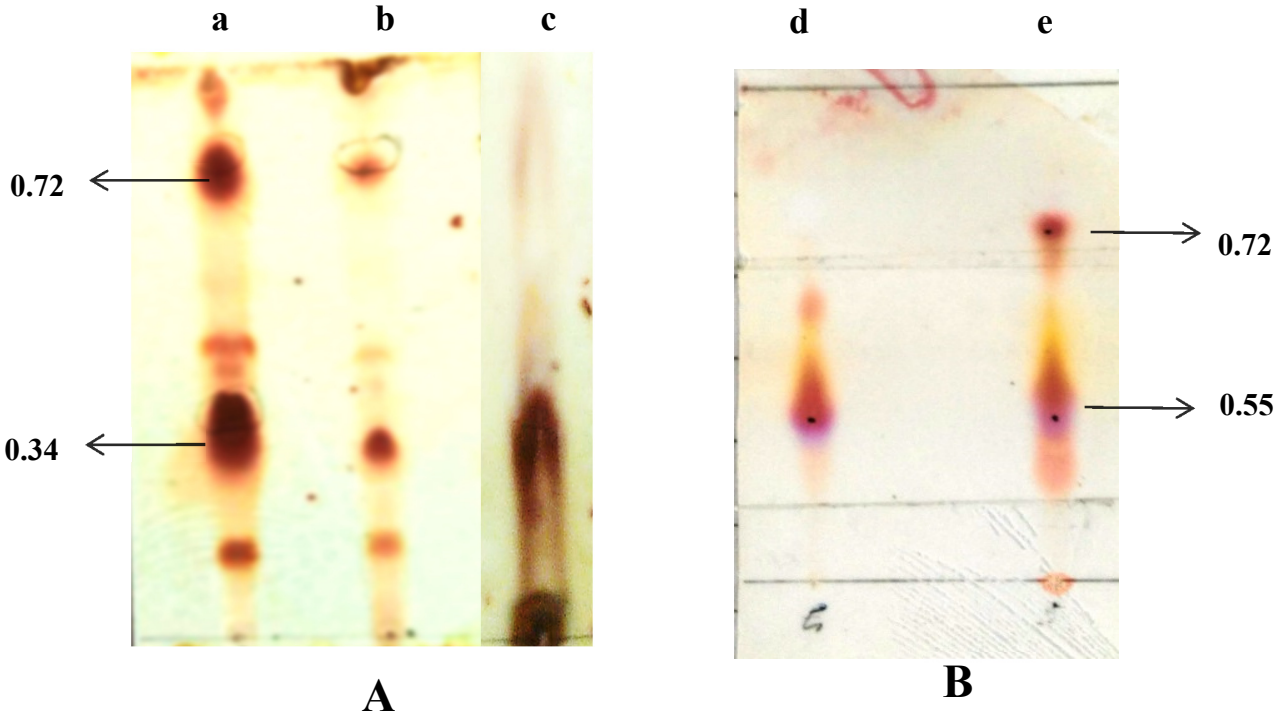

Table 1: Primary screening of isolates for biosurfactant production

Hydrocarbon Contaminated (Refinery) Samples														Extreme environments					
IOCL (Mathura)			IOCL (Guwahati)			BPCL (Mumbai)			IOCL (Bongaigaon)			Reliance (Jamnagar)			Hot spring (Manikaran)			Desert soil (Pokhran)		
	DCM	OSM		DCM	OSM		DCM	OSM		DCM	OSM		DCM	OSM		DCM	OSM		DCM	OSM
TC1	++	+	GR1	++	+++	PS1	+++	++++	BRSP1	++	+++	GRTL1	-	-	HPHS1	++	+++	SB2	+++	++++
PSMR1	++	+	GR2	++	++	PSD1	-	-	BRTF1	+++	+++	GART1	-	-	HPHS2	+	++	SB1	-	-
COMR1	++	+++	GR3	-	-	MANG1	++	++	BRAT1	+	++	GMEF1	+	++						
TC2	++++	++++	GRLSC1	++	+++	IPIG1	++	+++	BRRO1	+++	++++	GCHF1	-	-						
WDM1	+	++	GRAC1	++	+	MUCK1	++	++	BRAS1	++	++	GUCK1	++	+++						
WDM2	++++	++++	GREEO	-	-	CRTB1	-	-	BRTW1	++	++									
WDM4	++	++	GREW1	++++	++++	MUCK1	++	+++	BRSOIL	+++	++									
WDM3	-	-	GRAS1	++++	++++				SLDBR	++	+++									
MRBSIT1	+++	++++	GRDS1	++++	++++															
MRBSIT2	+	++	GRSOIL1	+++	+++															
MRBRPS2	-	-																		
MRBRPS1	++	+++																		
SLDB1	+++	++++																		

Drop collapse Method (DCM): (-) completely spherical: (+) flat: (++) moderately flat: (+++) completely flat.

Oil spread Method (OSM): (-) no displacement: (+) oil displacement < 1 cm in diameter: (++) 1 to 3 cm: (+++) 3-5 cm in diameter: (++++) > 5 cm in diameter.

Table 2: Qualitative and quantitative screening of the isolates for biosurfactant production

S.No	Isolates	Haemolytic assay (HA)	CTAB	Surface tension (mN/m)	Emulsification index (E_{24}) (%)	Emulsification activity (EU/mL)
1	*Achromobacter* sp. (PS1)	+ve (β- haemolysis)	+ve	30.43 ± 0.44	69.90 ± 0.97	97.05 ± 1.62
2	*Ochrobactrum* sp. (GREW1)	+ve (α-haemolysis)	-ve	31.14 ± 0.68	59.51 ± 1.22	56.87 ± 0.89
3	*Bacillus* sp. (SLDB1)	-ve (β-haemolysis)	-ve	31.10 ± 0.71	65.23 ± 1.48	91.38 ± 1.72
4	*Bacillus* sp. (SB2)	-ve (γ-haemolysis)	-ve	28.16 ± 0.19	61.35 ± 1.15	62.98 ± 2.29
5	*Pseudomonas* sp. (MRBSIT1)	+ve (β-haemolysis)	+ve	32.24 ± 1.04	64.22 ± 1.44	70.01 ± 0.52
6	*Achromobacter* sp. (WDM1)	+ve (β-haemolysis)	-ve	34.32 ± 0.59	51.34 ± 2.26	35.91 ± 2.14
7	*Bacillus* sp. (WDM2)	+ve (β-haemolysis)	-ve	42.85 ± 1.85	22.13 ± 2.18	30.22 ± 1.10
8	*Bacillus* sp. (TC2)	+ve (α-haemolysis)	-ve	37.28 ± 1.24	21.68 ± 2.61	16.03 ± 1.08
9	*Bacillus* sp. (GR1)	-ve (γ-haemolysis)	-ve	36.65 ± 2.43	51.36 ± 1.92	13.74 ± 1.97
10	*Lysinibacillus* sp. (GRAS1)	+ve (α-haemolysis)	-ve	35.82 ± 1.62	22.15 ± 2.21	86.23 ± 0.66
11	*Citrobacter* sp. (BRRO1)	+ve (α-haemolysis)	-ve	38.40 ± 1.58	30.37 ± 1.36	39.01 ± 0.72

***Control (Un-inoculated) (ST): 69.84 ± 2.2; Deionized water (ST): 70.31 ± 1.84**
The standard deviations presented are based on triplicate (SD, n = 3)

Table 3: 16S-rRNA sequence results of the potent eleven bacterial isolates

Isolates	Sample used for isolation	Bacterial isolate	Taxonomic affiliation (Class)	NCBI Accession numbers	16S-rRNA sequence comparison	
					Next relative by Genbank alignment	Similarity (%)
PS1	Oil spilled refinery soil	*Achromobacter sp.*	Betaproteobacteria	KT735240	*Achromobacter insolitus* (NR_025685)	99
GREW1	Refinery raw oil effluent	*Ochrobactrum sp.*	Alphaproteobacteria	KU321208	*Ochrobactrum tritici* (NR_114980)	100
SLDB1	Tank settled refinery sludge	*Bacillus sp.*	Firmicutes	KT735238	*Bacillus subtilis* (NR_113265)	99
SB2	Desert soil	*Bacillus sp.*	Firmicutes	KT735237	*Bacillus licheniformis* (NR_118996)	99
MRBSIT1	Bioremediation site soil	*Pseudomonas sp.*	Gammaproteobacteria	KU321209	*Pseudomonas aeruginosa* (NR_117678)	100
WDM1	Refinery waste drain	*Achromobacter sp.*	Betaproteobacteria	KT735239	*Achromobacter pulmonis* (NR_117644)	99
WDM2	Refinery waste drain	*Bacillus sp.*	Firmicutes	KT735241	*Bacillus subtilis* (NR_113265)	99
TC2	Refinery storage tank cleaning water	*Bacillus sp.*	Firmicutes	KT735242	*Bacillus subtilis* (NR_113265)	99
GR1	Refinery surge pond water	*Bacillus sp.*	Firmicutes	KT735243	*Bacillus aerius* (NR_118439)	99
GRAS1	Activated sludge	*Lysinibacillus sp.*	Firmicutes	KT735244	*Lysinibacillus fusiformis* (NR_112569)	99
BRRO1	Refinery recovered oil	*Citrobacter sp.*	Gammaproteobacteria	KT735246	*Citrobacter freundii* (NR_028894)	99

Table 4: Evaluation of the hydrocarbon degrading potential of the biosurfactant producing isolates

Isolate	Qualitative analysis DCPIP Method					Quantitative analysis Degradation of 2% w/v crude oil in MSM			
	Complex		Aromatic	Medium chain	Short chain	Biosurfactant production & concomitant hydrocarbon degradation		GC-FID results (% Area basis)	
	Crude	Diesel	Benzene	Decane	Hexane	Gravimetric analysis of TPH (%)	Surface tension (mN/m)	Aliphatic fraction	Aromatic fraction
Achromobacter sp. (PS1)	+++	++++	-	+	-	46.32 ± 4.44	32.43 ± 0.83	70.77	77.17
Ochrobactrum sp. (GREW1)	+++	++	-	+	-	38.93 ± 2.09	33.14 ± 0.54	79.24	72.13
Bacillus sp. (SLDB1)	+++	+++	-	+	-	22.58 ± 2.49	32.56 ± 0.22	87.65	67.70
Bacillus sp. (SB2)	++	+	-	-	+	20.91 ± 0.66	28.16 ± 1.16	82.47	73.88
Pseudomonas sp. (MRBSIT1)	+++	+++	+	-	+	25.19 ± 3.98	32.56 ± 0.83	93.86	41.56
Achromobacter sp. (WDM1)	+++	+++	-	-	-	17.50 ± 1.77	33.68 ± 1.44	50.34	49.02
Bacillus sp. (WDM2)	++	++	-	-	-	18.00 ± 2.22	42.85 ± 1.18	25.38	22.65
Bacillus sp. (TC2)	++	++	-	+	+	24.18 ± 1.10	32.37 ± 0.13	85.65	74.62
Bacillus sp. (GR1)	++	+++	-	-	-	23.56 ± 0.91	41.76 ± 0.52	43.90	49.25
Lysinibacillus sp. (GRAS1)	++	++	-	-	-	18.20 ± 2.24	58.30 ± 3.75	10.27	42.72
Citrobacter sp. (BRRO1)	+++	++	+	-	-	19.51 ± 2.09	45.42 ± 0.77	8.35	48.25

The symbols + and - indicate that the solution was colorless (degraded) or blue (not degraded), respectively (++++ colour change in 48h, +++ colour change in 72 h, ++ colour change in 96h, + colour change above 96 h); Control (Un-inoculated) (ST) – 59.27 ± 1.16 mN/m

The standard deviations presented are based on triplicate (SD, n = 3)

Table 5: Composition of fatty acids determined by GC-MS

Fatty acid composition	**Relative area percentage**		
Glycolipid	**Rhamnolipid (Jeneil)**	***Achromobacter*** **sp. (PS1)**	***Bacillus*** **sp. (SLDB1)**
Decanoic acid ($C_{10:0}$)	91.45 ± 0.04	88.27 ± 0.07	90.11 ± 0.07
Decenoic acid ($C_{10:1}$)	0.03 ± 0.07	-	-
Dodecanoic acid ($C_{12:0}$)	5.68 ± 0.03	8.25 ± 0.03	6.38 ± 0.04
Tertradecanoic acid ($C_{14:0}$)	0.59 ± 0.19	-	3.04 ± 0.08
Hexadecanoic acid ($C_{16:0}$)	0.71 ± 0.03	3.02 ± 0.01	-
Octadecenoic acid ($C_{18:1}$)	0.51 ± 0.09	-	-
Heneicosanoic acid ($C_{21:0}$)	0.39 ± 0.08	1.69 ± 0.04	-
Lipopeptide	**Surfactin (Sigma)**	***Ochrobactrum*** **sp. (GREW1)**	***Bacillus*** **sp. (SB2)**
Dodecanoic acid ($C_{12:0}$)	2.36 ± 0.06	-	4.93 ± 0.02
Tetradecanoic acid ($C_{14:0}$)	3.21 ± 0.01	1.44 ± 0.02	-
Hexadecanoic acid ($C_{16:0}$)	38.03 ± 0.06	1.26 ± 0.03	56.89 ± 0.01
Octadecanoic acid ($C_{18:0}$)	14.95 ± 0.01	33.03 ± 0.09	-
Octadecenoic acid ($C_{18:1}$)	-	14.84 ± 0.16	-
Nonadecanoic acid ($C_{19:0}$)	39.06 ± 0.06	-	-
Docosanoic acid ($C_{20:0}$)	-	17.24 ± 0.03	-
Heneicosanoic acid ($C_{21:0}$)	-	32.82 ± 0.03	39.02 ± 0.03

The standard deviations presented are based on triplicate (SD, n = 3)